IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| In re application of | : | |
| Takashi Shinohara et al. | : | |
| Application No. 10/553,118 | : | Group Art Unit 1632 |
| Filed on November 3, 2005 | : | Examiner: Ton, Thaian N. |

For: Method of growing sperm stem cells in vitro, sperm stem cells grown by the method, and medium additive kit to be used in growing sperm stem cells in vitro.

CORRECTED DECLARATION UNDER 37 CFR 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sirs:

I, Takashi SHINOHARA, a citizen of Japan residing at 23-6, Okazaki Tokusei-cho, Sakyo-ku, Kyoto 606-8351 Japan, sincerely declare that:

I am the same Takashi SHINOHARA that provided the DECLARATION UNDER 37 C.F.R. 1.132 dated February 27, 2009;

My earlier provided DECLARATION UNDER 37 C.F.R. 1.132 dated February 27, 2009, contained a typographical error that is being corrected by way of this CORRECTED DECLARATION UNDER 37 C.F.R. 1.132, which is intended to replace my earlier provided DECLARATION UNDER 37 C.F.R. 1.132 dated February 27, 2009;

I was born in August 1968 in Japan;

I graduated from the Faculty of Medicine, Kyoto University, Kyoto, Japan, with the degree of Doctor of Medicine in 1993;

I graduated from Graduate School of Medicine, Kyoto University, Kyoto, Japan, with the degree of Doctor of Philosophy in 1996;

I worked at Professor Ralph L. Brinster's Laboratory in University of Pennsylvania, PA, USA, as a Postdoctoral fellow from 1996 to 2000;

I was an Assistant Professor at Department of Medical Chemistry, Kyoto University Medical School, from 2000 to 2002;

I was an Associate Professor at Horizontal Medical Research Organization, Kyoto University Medical School, from 2003 to 2004;

I am a full Professor of the Department of Molecular Genetics, Kyoto University Medical School, since 2004;

I published with other research workers, reports on scientific studies, among others, including

1. β1- and α6-integrin are surface markers on spermatogonial stem cells. Proc. Natl. Acad. Sci. USA 96, 5504-5509, 1999;
2. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc. Natl. Acad. Sci. USA 97, 8346-8351, 2000;
3. Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc. Natl. Acad. Sci. USA 98, 6186-6191, 2001;
4. Adenovirus-mediated gene delivery and in vitro microinsemination produce offspring from infertile male mice. Proc. Natl. Acad. Sci. USA 99, 1383-1388, 2002;
5. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol. Reprod. 69, 612-616, 2003;
6. Generation of pluripotent stem cells from neonatal mouse testis. Cell 119, 1001-1012, 2004;
7. Production of knockout mice by random and targeted mutagenesis in spermatogonial stem cells. Proc. Natl. Acad. Sci. USA 103, 8018-8023, 2006;
8. The germ of pluripotency. Nat. Biotechnol. 24, 663-664. 2006; and
9. Adenovirus-mediated gene delivery into mouse spermatogonial stem cells. Proc. Natl. Acad. Sci. USA 104, 2596-2601, 2007;

I am an inventor of the above-identified U.S. Patent Application No. 10/553,118, which is assigned to Kyoto University; and

The following experiment was conducted under my supervision and direction, wherein the growth of spermatogonial stem cells in a medium containing GDNF and LIF was compared with that in a medium containing GDNF, LIF, bFGF and EGF.

## Experiment

***Experiment 1***

Mouse spermatogonial stem cells were established according to the method described in Example 1 in the specification of the above-identified U.S. patent application SN 10/553,118.

In brief, testis cells were collected by two-step enzymatic digestion from a newborn DBA/2 mouse.

Dissociated testis cells were allocated to gelatin-coated cell culture plate. Culture medium for the testis cells was "F/S medium" supplemented with 20 ng/ml mouse epidermal growth factor (EGF: Becton Dickinson), 10 ng/ml basic fibroblast growth factor (bFGF: Becton Dickinson), $10^3$ units/ml ESGRO (mouse leukemia inhibitory factor: LIF, Invitrogen) and 10 ng/ml recombinant rat GDNF (R&D Systems). The "F/S medium" used in this experiment was StemPro-34 SFM (Invitrogen) supplemented with StemPro supplement (Invitrogen), 25 μg/ml Insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 nM sodium selenite, 6 mg/ml D-(+)-glucose, 30 μg/ml pyruvic acid, 1 μl/ml DL-lactic acid (Sigma), 5 mg/ml bovine albumin (ICN Biomedicals), 2 mM L-glutamine, $5\times10^{-5}$ M 2-mercaptoethanol, MEM non-essential vitamin solution (Invitrogen), $10^{-4}$ M ascorbic acid, 10 μg/ml d-biotin, 30 ng/ml β-estradiol, 60 ng/ml progesterone (Sigma) and 1 (v/v)% fetal calf serum (JRH Biosciences). The cells were maintained on mitomycin C-inactivated mouse embryonic fibroblasts (MEF) and passaged every 3 to 5 days to fresh MEF at a one-third to one-fourth dilution at 37°C in an atmosphere of 5% carbon dioxide in air for about two months to establish spermatogonial stem cell colonies.

Established spermatogonial stem cells were dispersed by trypsin, transferred onto 6-well plate at 3 x $10^5$ cells per well and cultured at 37°C in a medium selected from following media A to C:

Medium A: F/S medium supplemented with 20 ng/ml mouse EGF, 10 ng/ml bFGF, $10^3$ units/ml mouse LIF and 10 ng/ml rat GDNF.

Medium B: F/S medium.

Medium C: F/S medium supplemented with $10^3$ units/ml mouse LIF and 10 ng/ml rat GDNF.

After 5 days, cells were treated with trypsin and the cells were counted. Results are shown in the following Table.

| | Medium A | Medium B | Medium C |
|---|---|---|---|
| Mean (cells) | 118 | 4.71 | 94.8 |
| S.E. | 5.69 | 0.567 | 7.87 |
| Mean (growth folds) | 3.92 | 0.1571 | 3.159 |
| S.E. | 0.1895 | 0.01890 | 0.2625 |

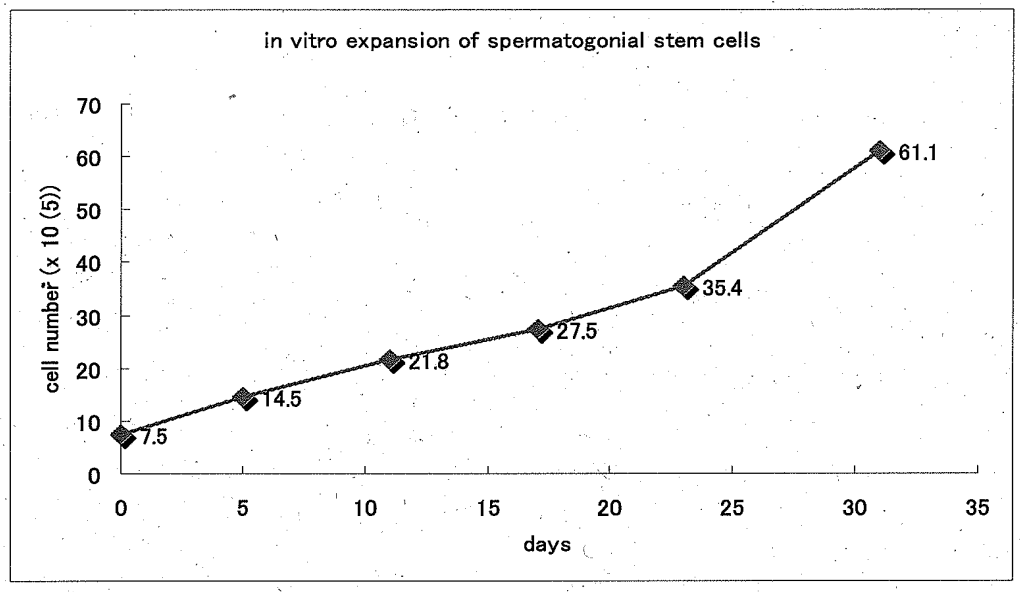
As shown in the Table, spermatogonial stem cells can grow in a medium containing GDNF and LIF and without any other growth factors, at a level similar to that when grown in a medium containing GDNF, LIF, EGF and bFGF.

***Experiment 2***

Mouse spermatogonial stem cells were established as described in Experiment 1.

Established spermatogonial stem cells were dispersed by trypsin treatment, transferred onto 6-well plate at $7.5 \times 10^5$ cells per well and cultured at 37°C in the F/S medium supplemented with $10^3$ units/ml mouse LIF and 10 ng/ml rat GDNF.

The number of spermatogonial stem cells was monitored sequentially for 30 days. Results are shown in the following graph.



As shown in the above graph, spermatogonial stem cells can grow in a medium containing GDNF and LIF and without any other growth factors, for 30 days.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed at Kyoto, Japan, on this date 9/3/09

Takashi SHINOHARA